AN ANTIBIOTIC PRODUCED BY THE MYCORRHIZAL FUNGUS CENOCOCCUM GRANIFORME[1]

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Received June 3, 1963

Abstract

An antibiotic active against representatives of the bacteria, yeasts, and actinomycetes has been extracted from the mycelium, produced in vitro, of several strains of the mycorrhizal fungus, *Cenococcum graniforme*. No activity was observed in culture fluids of this organism. The antibiotic differed from that found in *Boletus luteus* mycelium by its microbial inhibition and ultraviolet absorption spectra.

Introduction

Santoro and Casida (5) described antibiotics which occur in the mycelium of various laboratory cultures of *Boletus* and *Amanita* species of mycorrhizal fungi. The antibiotic from *Boletus luteus* was a polyene type, although it demonstrated antibacterial instead of the antifungal activity associated with other polyene antibiotics (2). Santoro (3) also observed that mycelium of the mycorrhizal fungus *Cenococcum graniforme* of the Fungi Imperfecti possibly possessed antibiotic activity but the study of this organism was not pursued further.

In nature, *Cenococcum graniforme* is present in mycorrhizae on the roots of trees growing in various poor forest soils, in which the basidiomycete mycorrhizae may be present only in low numbers, and also in better soils, where the latter mycorrhizae exhibit good numbers (1). Thus, the production of an antibiotic by this fungus would be of interest from an ecological standpoint in that it possibly could aid the mycorrhizae and hence the tree in competing with the soil microflora for nutrients and in preventing attack by soil-borne pathogens.

Materials and Methods

Cenococcum graniforme Strains

Seven strains of *Cenococcum graniforme* were examined. Strain A was obtained from Dr. E. Hacskaylo (Forest Physiology Laboratory, United States Department of Agriculture, Beltsville, Maryland) and the remaining strains from Dr. J. M. Trappe (Pacific Northwest Forest and Range Experiment Station, United States Department of Agriculture, Portland, Oregon). Stock cultures were maintained on Hoagland's amended medium (5) and were incubated and stored at 25° C. Transfers to fresh medium were made every 14 days.

Growth and Antibiotic Extraction

Fourteen-day mycelial inoculum grown by the bottle-bead technique of Santoro and Casida (4) was transferred to 2-liter Erlenmeyer flasks containing 300 ml Hoagland's amended medium adjusted to pH 4.0 with HCl, and the flasks were incubated 14 days at 25° C on a reciprocating shaker operating at 83 strokes per minute through a distance of 3¼ inches. The mycelium was

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[1] Authorized for publication on May 27, 1963, as paper No. 2785 in the journal series of the Pennsylvania Agricultural Experiment Station.

Canadian Journal of Microbiology. Volume 10 (1964)

separated from the culture fluid, which did not contain antibiotic, by filtration through cheesecloth, washed with distilled water, and squeezed by hand to remove excess moisture. The mycelium then was ground to a pasty slurry with sand in a mortar, and methanol or acetone was added to extract the antibiotic. Mycelial debris and sand were removed by filtration, and the extract, approximately pH 4.2, was adjusted to pH 5.0 and concentrated in a rotary solvent evaporator (Rinco Instrument Co., Greenville, Illinois). Mannitol, which also was extracted by these solvents, was allowed to crystallize from cold antibiotic solution and was removed by filtration.

Antibiotic Assay

Activity of antibiotic solutions was determined against *Bacillus cereus* using the standard filter paper disk assay procedure with nutrient agar to which had been added 0.1% glucose. The antibiotic solution was applied to the disks and allowed to air dry before the disks were placed on the seeded agar surface. Assay plates, thus prepared, were refrigerated 2 hours before incubation for 18 hours at 30° C.

Inhibition zone diameters and log concentrations of strain A antibiotic were plotted to give a standard curve of antibiotic activity. From this curve a zone diameter of 30 mm was arbitrarily chosen to represent 100 units of antibiotic activity.

The microbial inhibition spectrum of the antibiotic against a series of test organisms also was determined by the filter paper disk assay procedure.

Paper Chromatography

Antibiotic solutions were chromatographed by ascending and descending chromatography on Whatman No. 4 paper. Antibiotic activity on the dried chromatograms was located by the light bluish-green fluorescence of the compound under an ultraviolet source (Mineral Lamp—Ultra-Violet Products, Inc., South Pasadena, California) and by bioautograph plates with *Bacillus cereus* and a medium similar to that for antibiotic assay.

Results

Biological Properties

Extracts from mycelium of each of the strains of *C. graniforme* were tested for antibiotic activity against various bacteria, yeasts, actinomycetes, and fungi. Considering the variation in potencies of the antibiotic preparations tested, all antibiotic solutions exhibited similar inhibition spectra, see Table I. The antibiotic showed strong inhibition of Gram-positive bacteria and *Rhizobium meliloti*. At higher antibiotic concentrations activity was observed against other Gram-negative bacteria and against *Saccharomyces cerevisiae* and *Streptomyces griseus*; no inhibition was observed against the fungi listed in Table I.

Stability of Antibiotic

Ten milliliters portions of strain A antibiotic in acetone, which contained a small amount of water, were adjusted with 0.1 *N* KOH approximately to pH 5.0, 7.0, and 8.2. The antibiotic solutions in tightly stoppered tubes were heated for 30 minutes at 80° C then immediately cooled to 26° C. A portion of each sample was removed for determination of residual antibiotic activity

TABLE I

Microorganisms sensitive to antibiotic from various strains of *Cenococcum graniforme*

		Test microorganisms*									
C. graniforme strain	Activity, units†	*Bacillus cereus*	*B. mycoides*	*Staphylococcus aureus*	*Arthrobacter globiformis*	*Agrobacterium tumefaciens*	*Escherichia coli*	*Pseudomonas fluorescens*	*Rhizobium meliloti*	*Streptomyces griseus*	*Saccharomyces cerevisiae*
		Growth inhibition zones, mm									
A	34.0	23	25	23	25	23	14	16	32	18	17
	14.0	18	19	16	19	NA‡	NA	NA	20	NA	NA
1	12.0	17	17	18	18	NA	NA	NA	19	NA	NA
2	34.0	23	25	23	25	23	14	16	32	18	17
	16.5	19	18	19	21	NA	NA	NA	22	NA	NA
3	12.0	17	17	17	18	NA	NA	NA	19	NA	NA
4	9.5	15	15	16	17	NA	NA	NA	17	NA	NA
5	10.0	16	14	15	16	NA	NA	NA	18	NA	NA
7	8.7	15	14	16	16	NA	NA	NA	15	NA	NA

*No activity was noted against the following test microorganisms: *Pseudomonas aeruginosa*, 197*a*; *Aspergillus niger*; *Mucor* sp., 321; *Rhizopus* sp., 322; *Rhizoctonia solani*.

†Based on strain A antibiotic units.

‡NA = No activity.

against *B. cereus*, and the acetone solutions then were readjusted approximately to pH 6.0 with 0.1 *N* KOH or HCl and retested for antibiotic activity.

The antibiotic retained its activity after heating at pH 5.0 and 7.0 but did not demonstrate activity at pH 8.2 unless the pH was readjusted to 6.0 (Table II). However, in the latter instance only 86% of the activity was recovered.

The antibiotic in nonextracted mycelium also exhibited good stability. A 13.7 g sample of damp mycelium was dried at 80° C for 2 days then extracted with methanol to yield a total of 362 units of antibiotic activity. Similar mycelium, 12.4 g, which had not been oven-dried, yielded 392 units. When mycelium was oven-dried then ground with sand and extracted overnight in a Soxhlet apparatus with methanol, the antibiotic yield was approximately double that obtained by the usual methanol extraction procedure.

TABLE II

Heat and pH stability of *Cenococcum graniforme* antibiotic in acetone

	pH readjustment	
	None	To pH 6.0
Initial pH adjustment*	Residual antibiotic activity,† units/ml	
5.0	14.0	14.0
7.0	10.5	14.0
8.2	NA‡	12.0

*Unadjusted antibiotic solution was approximately pH 4.0 and contained 14.0 units/ml.

†After 30 minutes heating at 80° C.

‡NA = No activity.

TABLE III

Paper chromatography of antibiotic from *Cenococcum graniforme* strains

Solvent system No.	Solvent ratios	Type of chromatography	R_f values* for *Cenococcum graniforme* strains						
			A	1	2	3	4	5	7
1	Methanol, NH_4OH, H_2O 3:1:1	Ascending	0.84	0.84	0.76	0.79	0.79	0.75	0.78
2	Isopropanol, methanol, NH_4OH, H_2O 2:2:1:6	"	0.71	0.71	0.71	0.71	0.69	0.69	0.71
4	Isopropanol, methanol, pyridine, H_2O 2:2:1:6	"	0.87	0.87	0.85	0.82	0.84	0.82	0.84
11	Ethyl acetate, methanol, NH_4OH, H_2O 7:2:1:6 (lower phase)	Descending	0.74	0.77	0.76	0.76	0.71	0.71	0.74
15	Ethyl acetate, pyridine, H_2O 7:2:8 (lower phase)	"	0.80	0.83	0.80	0.85	0.82	0.84	0.81

*All antibiotic areas on the chromatograms fluoresced light bluish green with a mineral lamp ultraviolet light source.

Solubility

Initial studies showed that antibiotic activity could be extracted from *C. graniforme* mycelium with acetone, with methanol, and to a lesser extent with chloroform. No activity was extracted with water.

Five-milliliter portions of a methanol mycelial extract of strain A, each containing 288 antibiotic units at approximately pH 5.0, were dried on 20 g portions of Celite (Johns-Manville Co.) at room temperature. Each Celite preparation then was stirred at room temperature in 100 ml of solvent and filtered. The residue on the filter paper also was washed with 100 ml of solvent, and the combined wash and filtrate for each solvent was concentrated in a rotary evaporator to 5 ml. Assay against *B. cereus* revealed the following per cent recoveries of antibiotic: methanol, 61; ether, 61; ethyl acetate, 52; *n*-butanol, 38; benzene, 38; *n*-heptane, 28.

This information was utilized to purify the antibiotic 2.5-fold on an activity–dry weight basis. Thus, a methanolic mycelial extract was dried on Celite and subjected to continuous ether extraction in a Soxhlet apparatus. The ether solution was filtered, evaporated to dryness, and the residue dissolved in warm ethyl acetate and filtered.

Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum in methanol for preparations of *C. graniforme* antibiotic was determined in a Beckman DU spectrophotometer with methanol as a reference. The antibiotic preparations had been partially purified by successive extractions with ether and ethyl acetate and by methanol elution of activity from paper chromatograms which had been irrigated with the lower phases of solvent systems 13 and 15 (Table III). System 13 contained ethyl acetate, *n*-butanol, methanol, pyridine, H_2O: 5,1,1,1,8, respectively, and presented an R_f value of 0.80. Antibiotic preparations purified by these methods

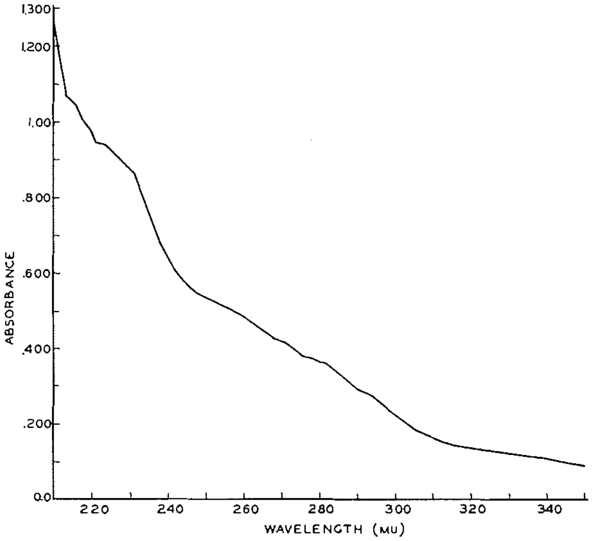

FIG. 1. Ultraviolet absorption spectrum for *C. graniforme* antibiotic preparation.

gave similar ultraviolet absorption curves, an example of which is shown in Fig. 1. It will be noted that no absorption peaks occurred in the range of 220 to 350 mμ. Similar trials have revealed no absorption peaks in the range from 206 to 440 mμ, indicating that the antibiotic is not of the polyene type.

Paper Chromatography

Acetone extracts of mycelium from the seven strains of *C. graniforme* were chromatographed with the solvent systems listed in Table III. Bioautographs of the chromatograph sheets (Table III) revealed that the mycelium of all strains contained similar antibiotic.

Discussion

The mycelium of each of the laboratory strains of *Cenococcum graniforme* which have been examined have contained the same antibiotic, and no antibiotic has been found free in the culture fluids of these organisms. The identity of this antibiotic was based on paper chromatographic evidence and the similarity of microbial inhibition spectra against a series of test organisms.

The *C. graniforme* antibiotic and the antibiotic (5) from another mycorrhizal fungus, *Boletus luteus*, both exhibit inhibitory activity directed mainly against bacteria. However, the bacterial inhibition spectra of the two antibiotics are not the same, and, in addition, the *C. graniforme* antibiotic possesses activity against representatives of the yeasts and actinomycetes. Neither antibiotic has demonstrated activity against filamentous fungi. Based on its ultraviolet absorption spectrum, the *B. luteus* antibiotic would appear to be a polyene (5) while that from *C. graniforme* does not reveal any defined absorption peaks in ultraviolet light.

The *C. graniforme* antibiotic was examined by paper chromatography at the Lilly Research Laboratories (Dr. R. Q. Thompson, Eli Lilly and Co., Indianapolis, Indiana) to determine its possible identity with antibiotics described in the literature. Comparison with 26 such antibacterial antibiotics produced by fungi and with Lilly and Company's spectrum of unnamed antibiotics revealed that the *C. graniforme* antibiotic has not previously been described.

In the present study, the *C. graniforme* antibiotic has been observed only in mycelium from pure cultures of the fungus. It would be of interest to determine whether this or other mycorrhizal fungus antibiotics also occur in natural mycorrhizal structures of these organisms. Such a determination should be facilitated by the black color and large radiating hyphae of *C. graniforme* mycorrhizae which allow identification of this particular association in nature.

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